

# Hispidulin, a small flavonoid molecule, suppresses the angiogenesis and growth of human pancreatic cancer by targeting vascular endothelial growth factor receptor 2-mediated PI3K/Akt/mTOR signaling pathway

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Hispidulin, an active component from *Artemisia vestita*, a traditional Tibetan medicinal plant, has been shown to possess anti-inflammatory and anti-oxidative activities. However, the functional role of hispidulin on tumor growth and angiogenesis has not been elucidated. We found that hispidulin significantly inhibited human pancreatic tumor growth in xenograft mice when s.c. treated at a dosage of 20 mg/kg daily, and this effect was accompanied with a potent inhibition on angiogenesis. When examining the cytotoxicity of hispidulin on HUVECs and pancreatic cancer cells *in vitro*, we found that HUVECs were more susceptible to the treatment, suggesting angiogenesis might be the primary target of hispidulin. Our results further showed that hispidulin inhibited vascular endothelial growth factor (VEGF)-induced cell migration, invasion, and capillary-like structure formation of HUVECs in a dose-dependent manner. In *ex vivo* and *in vivo* angiogenesis assays, we showed that hispidulin suppressed VEGF-induced microvessel sprouting of rat aortic rings and corneal neovascularization in C57/BL6 mice. To understand the underlying molecular basis, we next examined the effects of hispidulin on different molecular components in treated HUVECs, and found that hispidulin suppressed the VEGF-triggered activation of VEGF receptor 2, PI3K, Akt, mTOR, and ribosomal protein S6 kinase, but had little effect on focal adhesion kinase or extracellular signal-regulated kinase at an effective concentration. Taken together, our results indicate that hispidulin targets the VEGF receptor 2-mediated PI3K/Akt/mTOR signaling pathway in endothelial cells, leading to the suppression of pancreatic tumor growth and angiogenesis. (*Cancer Sci* 2011; 102: 219–225)

Cancer of the exocrine pancreas is characterized by extensive local invasion and metastases to the liver, and this aggressive biology translates into a 5-year survival rate of 1–4% for all patients with a diagnosis of pancreatic adenocarcinoma.<sup>(1)</sup> The current best therapy including surgery, radiation, and chemotherapy has done little to alter the cancer-related deaths of these patients.<sup>(2)</sup> As one of the hallmarks of cancer, angiogenesis is an essential event involved in tumor progression and metastasis.<sup>(3)</sup> Antiangiogenic therapy is now considered to be a prospective strategy because endothelial cells are genetically stable, which makes them an ideal therapeutic target compared with cancer cells.<sup>(4)</sup>

In the process of tumor angiogenesis, vascular endothelial growth factor (VEGF) plays a pivotal role in the regulation of

endothelial cell proliferation, migration, and vascular permeability through binding to its receptor tyrosine kinases such as VEGFR1, R2, and R3.<sup>(5,6)</sup> However, the VEGF signaling involved in angiogenesis is mainly mediated by VEGFR2.<sup>(7)</sup> Specifically, VEGFR2 activation leads to the activation of diverse intracellular signaling, and the PI3K/Akt signaling pathway is one of them. In angiogenesis, PI3K/Akt kinases are activated by a variety of stimuli in endothelial cells<sup>(8)</sup> and regulate multiple critical steps by phosphorylating different downstream substrates, such as the mTOR.<sup>(9)</sup> It has been well documented that mTOR kinase, a central regulator of cell metabolism, growth, proliferation, and survival,<sup>(10,11)</sup> is activated during various cellular processes such as tumor initiation, progression, and angiogenesis.<sup>(12,13)</sup> These observations have attracted broad scientific and clinical interest in the mTOR signaling pathway. However, with an increasing understanding of feedback loops exiting in the PI3K/Akt/mTOR pathway, it has been recognized that inhibition on one output is at the expense of activation of the others.<sup>(14)</sup> For this reason, there is a growing consensus that the inhibition of combined components in the PI3K/Akt/mTOR pathway likely reflects a mechanistic rationale for therapeutic options.<sup>(15,16)</sup>

Hispidulin, extracted from *Artemisia vestita*, a traditional Tibetan medicinal plant, has been used traditionally in China for treating various inflammatory diseases.<sup>(17)</sup> It has been reported that hispidulin is a partial positive allosteric modulator at the benzodiazepine receptor.<sup>(18,19)</sup> Given orally, it reduces seizures in a gerbil model of epilepsy.<sup>(20)</sup> Hispidulin also shows antifungal,<sup>(21)</sup> antiproliferative,<sup>(22)</sup> antioxidant,<sup>(23)</sup> and antithrombosis properties.<sup>(24)</sup> Very recent studies showed that hispidulin could potentiate the apoptosis of human ovarian cancer cells and human glioblastoma multiforme cells.<sup>(25,26)</sup> However, whether hispidulin has the ability to modulate tumor growth and tumor angiogenesis is still unknown. In this study, we investigated the functional roles of hispidulin in preventing pancreatic cancer growth in xenograft mice, examined the potential molecular targets by inhibiting tumor angiogenesis, and elucidated the signaling pathways mediated by hispidulin in endothelial cells.

## Materials and Methods

**Reagents.** Hispidulin was purchased from Shanghai Tauto Biotech (Shanghai, China) (purity > 98%) and its chemical

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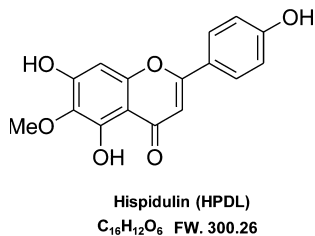


Fig. 1. Chemical structure of hispidulin.

structure is shown in Figure 1. A 100-mmol/L stock solution of hispidulin was prepared and stored at  $-20^{\circ}\text{C}$  as small aliquots until needed. Bacteria-derived recombinant human VEGF (VEGF<sub>165</sub>) was a gift from the Experimental Branch of the National Institutes of Health (Bethesda, MD, USA). Growth factor-reduced Matrigel was purchased from BD Biosciences (San Jose, CA, USA). Antibodies against Akt, mTOR, S6K, ERK, FAK, Bcl-2, Bcl-xL, Survivin, c-Myc, VEGFR2, PI3K,  $\beta$ -actin, and phospho-specific anti-Akt (Ser<sup>473</sup>), anti-mTOR (Ser<sup>2448</sup>), anti-S6K (Thr<sup>389</sup>), anti-ERK (Thr<sup>202</sup>/Tyr<sup>204</sup>), anti-FAK (Tyr<sup>397</sup>), anti-VEGFR2 (Tyr<sup>1175</sup>), and anti-PI3K (Tyr<sup>458</sup>) were purchased from Cell Signaling Technology (Danvers, MA, USA). The VEGFA antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Cell lines and cell culture.** Human umbilical vascular endothelial cells were purchased from ScienCell (Carlsbad, CA, USA) and cultured in endothelial cell medium (ECM; M199 served as the basal medium). Human pancreatic cancer cells (PANC-1, PANC-28 and BxPC-3) were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% fetal bovine serum. MCDB-131 medium was purchased from Invitrogen (Carlsbad, CA, USA). Cells were cultured at  $37^{\circ}\text{C}$  under a humidified 95%:5% (v/v) mixture of air and  $\text{CO}_2$ .

**Tumor xenograft study.** PANC-1 cells were detached by trypsinization, washed and resuspended in cold PBS. Five-week-old male BALB/cA nude mice were given an s.c. injection of  $5 \times 10^6$  cells into the right flank. After tumor sizes reached approximately  $50 \text{ mm}^3$ , mice were divided into two groups and treated with or without hispidulin at a dosage of 20 mg/kg/day for 35 days. Hispidulin was dissolved in minimum DMSO and injected s.c. around the solid tumors. The body weight of tumor-bearing mice was recorded every 5 days and tumor volume was calculated according to the formula  $A \times B^2 \times 0.5$ , where  $A$  is the longest diameter of the tumor and  $B$  is the shortest.

**Histology and immunohistochemistry.** Solid tumors fixed in 10% buffered formalin for 12 h were processed conventionally for paraffin-embedded tumor sections ( $5\text{-}\mu\text{m}$  thick). A blood vessel staining kit (von Willebrand factor; Millipore, Billerica, MA, USA) was used to indicate blood vessel formation and specific VEGF antibody was used to detect the expression in solid tumors.

**Cell viability assay.** Human umbilical vascular endothelial cells, PANC-1, PANC-28, and BxPC-3 cells ( $5 \times 10^3$  per well) were treated with various concentrations of hispidulin for 48 h in a 96-well plate. To determine the cell viability, a CellTiter 96 Aqueous One Solution Cell Proliferation kit [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS); Promega, Madison, WI, USA] and a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) were used.

**Cell migration assay.** Cell migration assay was carried out as described previously.<sup>(27)</sup> The HUVECs were allowed to grow to full confluence in a six-well plate, wounded with tips, then washed with PBS. The cells were treated with indicated con-

centrations of hispidulin and 50 ng/mL VEGF. The migrated endothelial cells were photographed using an inverted microscope after 8–12 h incubation at  $37^{\circ}\text{C}$  in a 95%:5% (v/v) mixture of air and  $\text{CO}_2$ . Three independent experiments were carried out.

**Transwell migration assay.** Transwell migration assay was carried out with 6.5 mm diameter polycarbonate filters ( $8\text{-}\mu\text{m}$  pore size) pre-coated with 0.1% gelatin. The HUVECs ( $4 \times 10^4$  per well) were first seeded into the upper chambers of the Transwell plates, then treated with various concentrations of hispidulin in 0.5% FBS ECM, while the bottom chambers were filled with 600  $\mu\text{L}$  of 0.5% FBS ECM plus 30 ng/mL VEGF. After 4–6 h incubation, the cells spreading on the upper surfaces of the filter (non-migrated cells) were wiped away with cotton swabs, and the migrated cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Images were taken by an inverted microscope and migrated cells were quantified by manual counting.

**Tube formation assay.** Tube formation assay was carried out as described previously.<sup>(28)</sup> A 96-well plate was precoated with growth factor-reduced Matrigel (50  $\mu\text{L}$  per well) and incubated at  $37^{\circ}\text{C}$  for 30 min. The HUVECs ( $2 \times 10^4$ /well) suspended in 0.5% FBS ECM were dispensed into each well and incubated with different concentrations of hispidulin and 30 ng/mL VEGF for 6–8 h. Capillary structure formation was photographed and quantified by measuring the tube length. Three independent experiments were carried out.

**Rat aortic ring assay.** Rat aortic ring assay was carried out as described previously.<sup>(29)</sup> In brief, a 48-well plate was coated with Matrigel and incubated at  $37^{\circ}\text{C}$  for 30 min. The 1.0–1.5 mm aortic rings, derived from the thoracic aorta of 5-week-old male rats, were randomized into wells and sealed with another 100  $\mu\text{L}$  overlay of Matrigel. Serum-free MCDB-131, containing 50 ng/mL VEGF, was added into plates with or without hispidulin. The fresh medium was changed every other day. At day 9, microvessel sprouts were photographed using an inverted microscope.

**Mouse corneal micropocket assay.** The mouse corneal assay was carried out according to procedures described previously.<sup>(30)</sup> Briefly, corneal micropockets were created with a needle of 1-mL injector in 5-week-old C57/BL6 mice. A micropellet of sucrose aluminum sulfate and hydron polymer containing 100 ng VEGF with or without 15  $\mu\text{g}$  hispidulin was plated into each corneal pocket. Eyes were photographed by a slit-lamp biomicroscope on day 6 after micropellet implantation. Maximal vessel length and clock hours of circumferential neovascularization were measured and vessel area was calculated according to the formula  $0.2\pi \times \text{length} \times \text{clock number}$ .

**Western blot analysis.** To explore the molecular mechanism of hispidulin on angiogenesis, we used Western blot analysis to detect key proteins involved in the biological functions of endothelial cells. The HUVECs were starved in 0.5% FBS ECM for 3–4 h, then pretreated with or without various concentrations of hispidulin for further 1–2 h, followed by simulation with 50 ng/mL VEGF for 2–20 min. As for survival-related protein detection, HUVECs and PANC-1 cells were directly treated with various concentrations of hispidulin for 48 h. The whole-cell extracts were prepared in RIPA buffer (20 mM Tris, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mM NaF, 10 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , and 1 mM phenylmethylsulphonyl fluoride) supplemented with proteinase inhibitor cocktail (Calbiochem, San Diego, CA, USA). Forty micrograms of cellular protein from each treatment was applied to 6–12% SDS-polyacrylamide gels and probed with specific antibodies, followed by exposure to HRP-conjugated goat anti-mouse or goat anti-rabbit antibody (Cell Signaling Technology). Protein concentrations were determined using bicinchoninic acid assay and equalized before loading.

**Statistics.** All data are presented as the mean  $\pm$  SD and statistical comparisons between groups were carried out using one-way ANOVA followed by Student's *t*-test. *P*-values  $\leq 0.05$  were considered statistically significant.

## Results

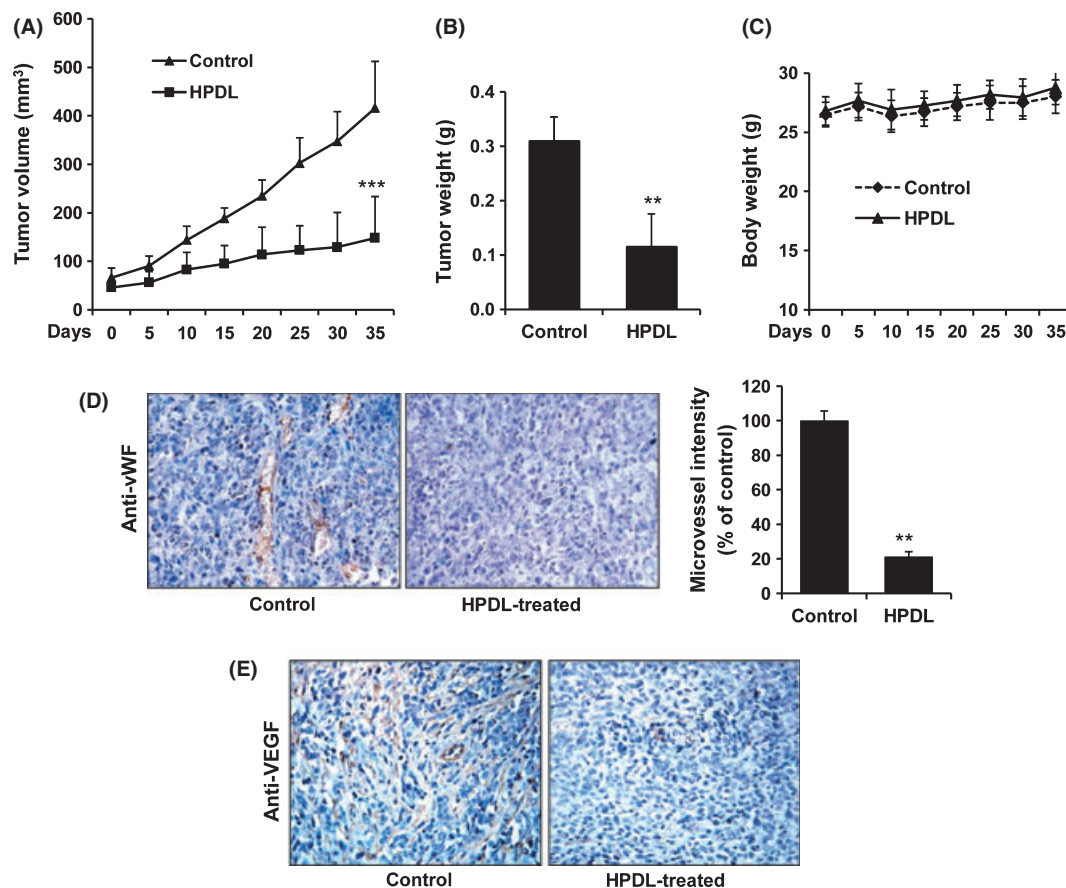
**Hispidulin inhibits tumor growth and tumor angiogenesis in xenograft mice.** To determine whether hispidulin (Fig. 1) exerts inhibitory effects on tumor growth, we set up a xenograft human pancreatic tumor mouse model. Our results revealed that s.c. treatment with 20 mg/kg hispidulin for 35 days significantly suppressed tumor volume (Fig. 2A) and tumor weight (Fig. 2B), but had little effect on mouse body weight (Fig. 2C). These data indicated that hispidulin had the ability to suppress tumor growth *in vivo*.

To further examine whether the inhibitory effect of hispidulin on tumor growth is through angiogenesis suppression, we examined the distribution of blood vessels in solid tumors with or without treatment with hispidulin using immunohistochemistry. We found that the intensity of blood vessels in the treated group was dramatically lower than that of the control (Fig. 2D), indicating that hispidulin inhibited tumor growth through suppression of angiogenesis. We also tested VEGF production in solid tumor, and the results showed that the expression of VEGF in the hispidulin-treated group was much lower than that of the controls (Fig. 2E).

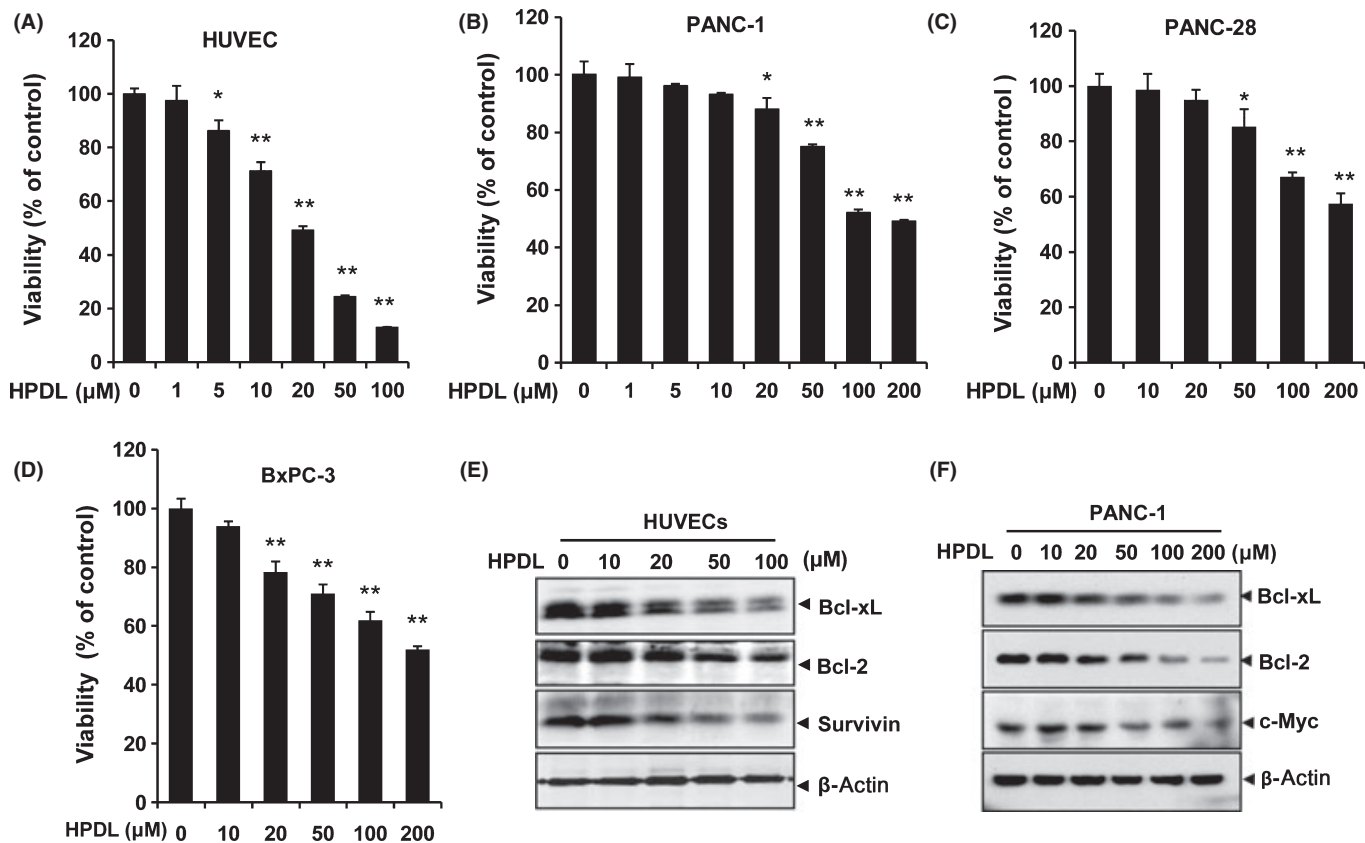
**Endothelial cells more sensitive to hispidulin treatment.** To investigate the effect of hispidulin on the viability of endothelial cells and pancreatic cancer cells, MTS assay was carried out. The results showed that the IC<sub>50</sub> of hispidulin on HUVECs was approximately 20  $\mu$ mol/L (Fig. 3A), versus IC<sub>50</sub> values of approximately 200  $\mu$ mol/L in PANC-1 (Fig. 3B), PANC-28 (Fig. 3C), and BxPC-3 cells (Fig. 3D), suggesting that endothelial cells were more susceptible to treatment. Similarly, survival-related proteins such as Bcl-xL, Bcl-2, c-Myc, and Survivin were also inhibited by hispidulin in HUVECs (Fig. 3E) and PANC-1 cells (Fig. 3F) and, consistently, the effective concentration of hispidulin was relatively lower in HUVECs.

**Hispidulin inhibits VEGF-induced cell migration and capillary structure formation of endothelial cells.** Endothelial cell migration and tube formation are essential steps for angiogenesis. To test whether hispidulin could affect the biological functions of endothelial cells, we investigated the inhibitory effects of hispidulin on VEGF-induced cell motility using a wound-healing migration assay (Fig. 4A) and Transwell migration assay (Fig. 4B). We found that hispidulin could inhibit cell motility in a dose-dependent manner. The effective concentration was approximately 10  $\mu$ mol/L.

Furthermore, we used a 2D Matrigel assay to test the effect of hispidulin on the tube formation of HUVECs. Our data showed that hispidulin inhibited VEGF-triggered tubular formation of endothelial cells in a dose-dependent manner, and 10  $\mu$ mol/L hispidulin could significantly decrease tube length (Fig. 4C).



**Fig. 2.** Hispidulin (HPDL) inhibits tumor growth and tumor angiogenesis in xenograft mice. PANC-1 pancreatic cancer cells were injected into nude mice ( $5 \times 10^6$  per mouse). After the tumor volume reached  $\sim 50$  mm<sup>3</sup>, the mice were s.c. treated with or without hispidulin at a dosage of 20 mg/kg daily. (A) Hispidulin inhibited tumor volume. (B) Tumor weight was significantly suppressed by hispidulin. (C) Hispidulin had no toxic effect on mouse body weight at tested dosages and conditions. (D) Hispidulin suppressed tumor angiogenesis. The 5- $\mu$ m sections of the solid tumor were stained using von Willebrand factor (vWF). Arrows indicate blood vessels. The vessel density was calculated statistically using Image-Pro Plus 6.0 software (Media Cybernetics Inc., Bethesda, MD, USA). (E) Hispidulin suppressed vascular endothelial growth factor (VEGF) expression in solid tumors. Columns or dots, mean; bars, SD; *P* < 0.01 vs control.



**Fig. 3.** Hispidulin (HPDL) inhibits cell viability in HUVECs and pancreatic cancer cells. The HUVECs and several pancreatic cell lines were treated with various concentrations of hispidulin for 48 h, followed by MTS assay, and cell survival-related proteins were also tested by Western blot assay. Hispidulin inhibited the viability of HUVECs (A), PANC-1 cells (B), PANC-28 cells (C), and BxPC-3 cells (D). (E) Hispidulin also suppressed the expression of Bcl-2, Bcl-xL, and survivin in HUVECs. Endothelial cells were directly treated with different concentrations of hispidulin for 48 h, and protein was examined by Western blotting. (F) Hispidulin suppressed the expression of Bcl-2, Bcl-xL, and c-Myc in PANC-1 cells. Pancreatic cancer cells were directly treated with hispidulin for 48 h. Columns, mean; bars, SD. \* $P < 0.05$ ; \*\* $P < 0.01$  vs control.

Together, these results indicated that hispidulin suppressed angiogenesis *in vitro*.

**Hispidulin inhibits microvessel sprouting *ex vivo* and VEGF-induced corneal neovascularization *in vivo*.** To evaluate the potential effect of hispidulin on angiogenesis, two well-established angiogenesis models were used *ex vivo* and *in vivo*. We determined the effects of hispidulin on microvessel sprouting *ex vivo* using the aortic ring assay. Our results showed that hispidulin dramatically inhibited VEGF-induced sprouting from the aortic rings (Fig. 5A), and 50 μmol/L of hispidulin almost completely inhibited the sprouting (Fig. 5B).

Furthermore, in the mouse corneal assay, 100 ng VEGF could significantly induce neovascularization in the cornea of C57/BL6 mice, whereas treatment with 15 μg of hispidulin potently inhibited VEGF-induced neovascularization (Fig. 6A). Maximal vessel length (Fig 6B,B1) and clock hours of circumferential neovascularization (Fig. 6B,B2) were consistently inhibited by hispidulin. Additionally, the blood vessel area in treated mice was smaller than that of controls (Fig. 6B,B3), suggesting that hispidulin suppressed VEGF-induced angiogenesis *in vivo*.

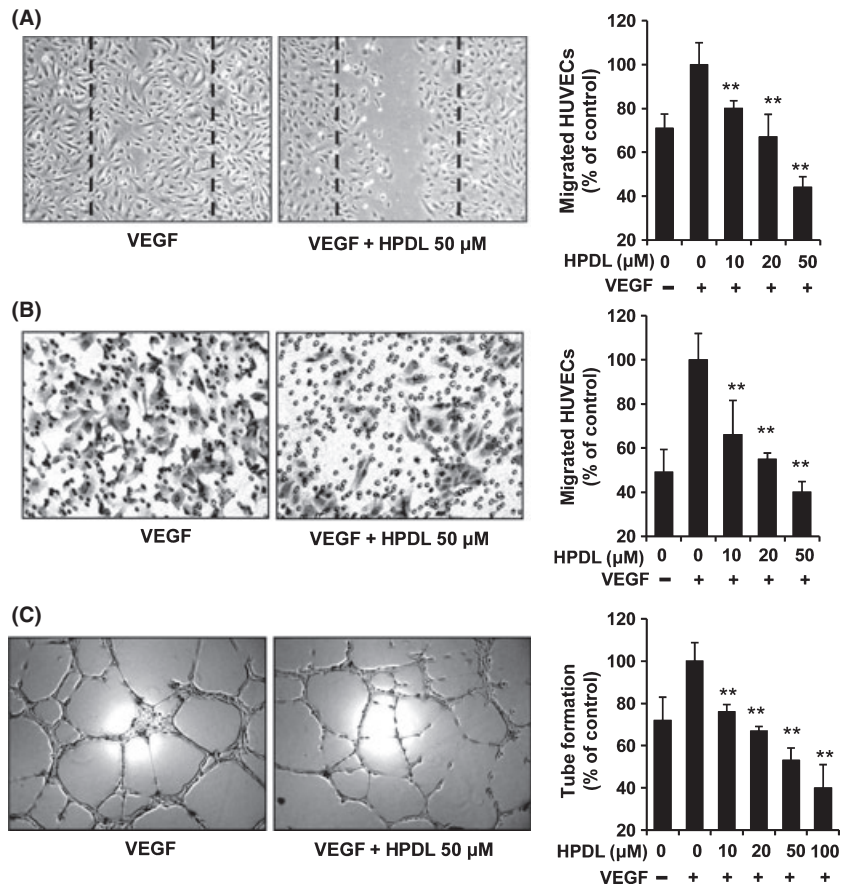
**Hispidulin inhibits VEGFR2-mediated signaling pathway.** To understand the molecular basis of hispidulin-mediated anti-angiogenic effects, we next examined the signaling pathways in treated HUVECs. Accumulating evidence shows that VEGFR2 is the crucial and main receptor mediating angiogenic and vascular permeability activity, thus we examined the action of hispidulin on the phosphorylation of VEGFR2. The results showed that hispidulin potently inhibited the activity of

VEGFR2 at 10 μmol/L (Fig. 7A), which was consistent with its *in vitro* functions. We also found that hispidulin significantly suppressed the activation of PI3K and Akt kinase (Fig. 7B), but not extracellular signal-regulated kinase and focal adhesion kinase (Fig. 7C). As a result of PI3K/Akt inhibition, the activation of mTOR and S6K kinase were blocked. These data indicated that hispidulin exerted its anti-angiogenic function primarily through blockade of the VEGFR2-mediated PI3K/Akt/mTOR pathway (Fig. 7D).

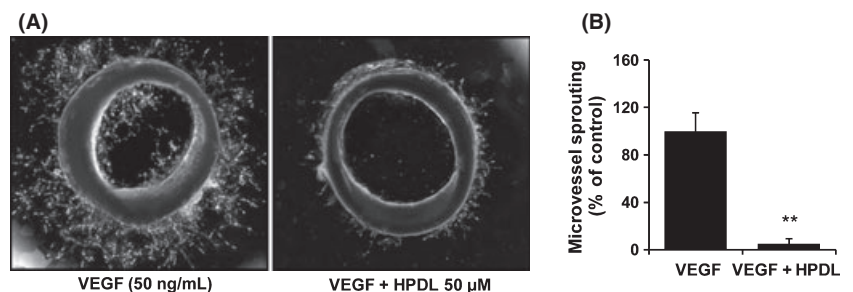
## Discussion

In this study, we showed for the first time that hispidulin, an active flavonoid from *Artemisia vestita*, has a potent ability to suppress human pancreatic tumor growth, an effect achieved to a great extent by angiogenesis inhibition.

In xenograft pancreatic tumor mice, 20 mg/kg/day hispidulin significantly suppressed tumor volume and tumor weight (Fig. 2A,B), without toxic effects on mouse body weight (Fig. 2C). Immunohistochemistry results further revealed that the generation of new blood vessels and the expression of VEGF in the treated group were dramatically reduced when compared with the control groups (Fig. 2D,E). However, whether this phenomenon occurs through effects on the biological function of endothelial cells or cancer cells, or both, was not ascertained. Therefore, we tested the effect of hispidulin on cell viability. Interestingly, we found that the IC<sub>50</sub> value of hispidulin in HUVECs was 20 μmol/L, whereas at least 200 μmol/L hispidulin



**Fig. 4.** Hispidulin (HPDL) inhibits vascular endothelial growth factor (VEGF)-induced cell migration and capillary structure formation of endothelial cells. (A) Hispidulin inhibited HUVEC migration. Cells were grown into full confluence in six-well plates and treated with indicated concentrations of hispidulin and VEGF. The migrated cells were quantified by manual counting. (B) Hispidulin inhibited HUVEC Transwell migration. Cells were seeded in the upper chamber of a Transwell and treated with hispidulin. After 4 h, the number of HUVECs that migrated through the membrane was quantified. (C) Hispidulin inhibited VEGF-induced tube formation of HUVECs. Cells were placed in 96-well plates coated with Matrigel. After 6–8 h, tubular structures were photographed in the absence and presence of hispidulin. Columns, mean; bars, SD. \*\* $P < 0.001$  vs control.



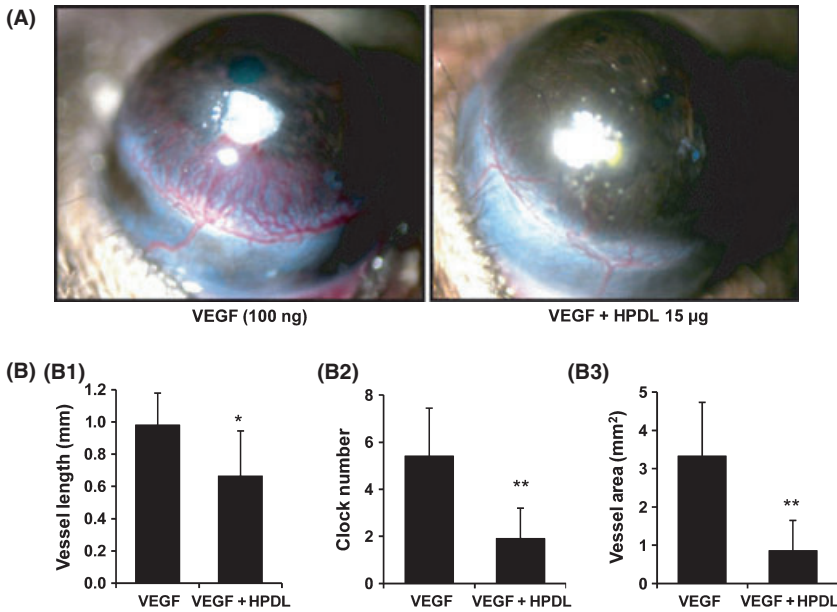
**Fig. 5.** Hispidulin (HPDL) inhibits vascular endothelial growth factor (VEGF)-induced microvessel sprouting *ex vivo*. Rat aortic rings were placed in 96-well plates precoated with Matrigel and treated with VEGF in the presence or absence of hispidulin for 9 days. (A) Photographs of endothelial cell sprouts from the aortic rings without or with treatment with hispidulin. (B) Sprouts were recorded using Image-Pro Plus 6.0 software and were calculated as a percentage of control groups. Columns, mean; bars, SD. \*\* $P < 0.01$  vs control.

was required to effectively inhibit cell viability in pancreatic cancer cells (PANC-1, PANC-28, and BxPC-3) (Fig. 3B–D), suggesting that endothelial cells were probably the primary target of hispidulin in tumor inhibition. In addition, hispidulin potently inhibited VEGF-induced microvessel sprouting *ex vivo* and corneal neovascularization *in vivo*. All of these results indicated that hispidulin is an angiogenesis inhibitor, and blocks multiple steps of angiogenesis.

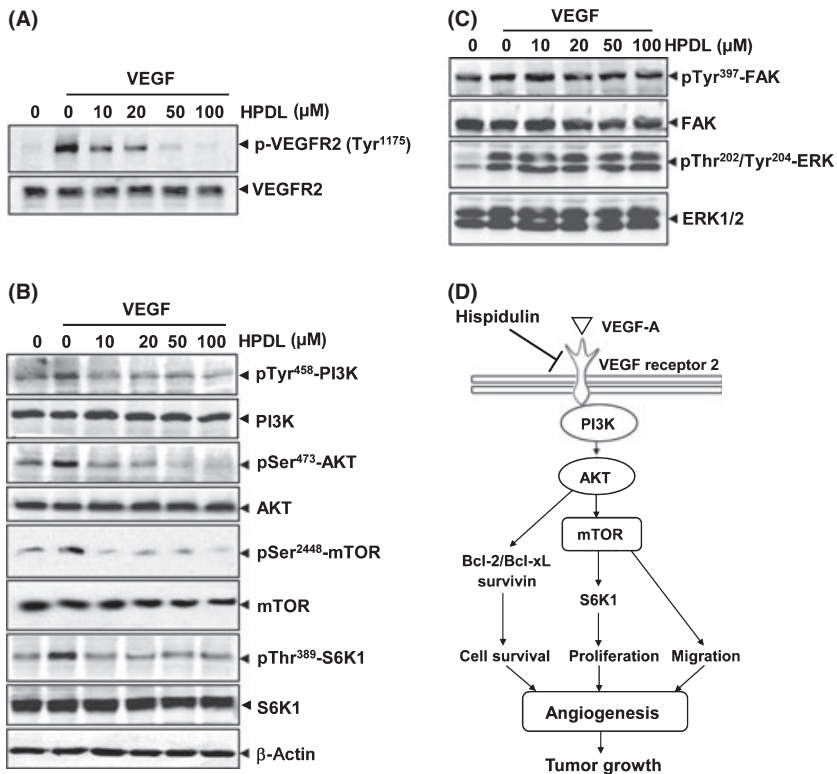
Accumulating evidence shows that VEGFR2 is the crucial and main receptor mediating angiogenic and vascular permeability activity, whereas VEGFR3 is mainly involved in lymph-angiogenic activity.<sup>(7)</sup> In this study, we found that hispidulin is a VEGFR2 inhibitor. The inhibitory effect of hispidulin on the activation of VEGFR2 was consistent with that on PI3K/Akt/mTOR signaling components. This indicates that VEGFR2 might be the important target of hispidulin in endothelial cells (Fig. 7D). Conversely, VEGFR1 contains a classical tyrosine kinase domain, however, the primary function of VEGFR1 may be as a negative regulator in vascular development.<sup>(31)</sup> Our

results showed that hispidulin significantly suppressed the VEGFR2-mediated PI3K/Akt/mTOR signaling pathway in endothelial cells at 10 μmol/L, which was in accord with the *in vitro* results that indicated hispidulin could block VEGF-induced endothelial cell migration, invasion, and tubular formation. mTOR kinase, a central regulator of cell metabolism, growth, proliferation, and survival,<sup>(10,11)</sup> has been implicated in inflammation by regulating signal-dependent translation in platelets, monocytes, and neutrophils.<sup>(32,33)</sup> A recent study also showed that PI3K/mTOR was an important target for leptin-induced formation of cytoplasmic lipid bodies, enhancing inflammatory mediator production in macrophages.<sup>(34)</sup> Our results indicated that hispidulin significantly inhibited mTOR kinase and its downstream target, S6 kinase (Fig. 7B), which might provide the reason that hispidulin exerted anti-inflammatory properties in previous reports.<sup>(17)</sup> However, how hispidulin affects the complex of mTOR kinase is still an open question.

It is documented that PI3K/Akt favors survival through the direct regulation of apoptotic proteins, such as Bcl-2 and



**Fig. 6.** Hispidulin (HPDL) inhibits vascular endothelial growth factor (VEGF)-induced corneal neovascularization *in vivo*. Micropellets containing 100 ng VEGF with or without 15 µg hispidulin were seeded into mouse corneal pockets. (A) Hispidulin inhibited VEGF-induced neovascularization. The photographs of mouse cornea were taken in the absence or presence of hispidulin. (B) Maximal vessel length and clock hours of circumferential neovascularization were recorded and the blood vessel area was calculated in hispidulin-treated and control groups. Columns, mean; bars, SD. \* $P < 0.05$ ; \*\* $P < 0.01$  vs control.



**Fig. 7.** Molecular basis of hispidulin (HPDL) in angiogenesis. (A) Hispidulin inhibited the activation of vascular endothelial growth factor receptor 2 (VEGFR2) at 10 µmol/L. (B) Hispidulin inhibited the activation of PI3K, Akt, mTOR, and S6 kinase. Proteins from different treatments were analyzed by Western blotting assay. (C) Hispidulin had little effect on the activation of FAK and ERK kinases. (D) Potential anti-angiogenic signaling pathways regulated by hispidulin in endothelial cells.

Bcl-xL.<sup>(35,36)</sup> Also, Bcl-2, Bcl-xL, and survivin are nuclear factor-κB regulated gene products and PI3K/Akt can also activate nuclear factor-κB.<sup>(37)</sup> In this study, we found that hispidulin could inhibit the expression of these cell growth-related proteins in a concentration-dependent manner in both endothelial cells and pancreatic cancer cells (Fig. 3E,F). Recently, endothelial p70S6K has been shown to play an important role in regulating tumor microenvironment and angiogenesis,<sup>(38)</sup> and our results showed that hispidulin could significantly decrease the activation of S6K; the effective

concentration of hispidulin is approximately 10 µmol/L. Consistent with the inhibition of these kinases at the molecular level, angiogenesis was dose-dependently rectified by hispidulin.

In summary, we show for the first time that hispidulin inhibited human pancreatic tumor angiogenesis and tumor growth by targeting the VEGFR2-mediated PI3K/Akt/mTOR signaling pathway. We have reason to believe that hispidulin could be a potential drug candidate for cancer prevention and cancer therapy.

## Acknowledgments

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## Disclosure Statement

No potential conflicts of interest were disclosed.

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